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Antirespiratory and Antiproliferative Activity of Anthralin in Cultured Human Keratinocytes

UWE REICHERT, PH.D., YANNICK JACQUES, PH.D.,* MICHELLE GRANGERET, AND RAINER SCHMIDT, PH.D.

Centre International de Recherches Dermatologiques (CIRD), Sophia Antipolis, Valbonne, France

The effect of anthralin and its oxidation products, anthralin-dimer and anthralin-quinone, on protein content and thymidine incorporation as well as CO_2 production from glucose and glutamine has been studied in transformed human keratinocytes in vitro. Anthralin exhibited the strongest inhibition, the dimer was generally less active and the quinone inactive. Respiration and thymidine incorporation were the most sensitive cellular functions showing 50% inhibition at about 1 and 3 μM anthralin, respectively. Comparison of the inhibition kinetics of anthralin with those of antimycin A and mitomycin C showed that anthralin behaved as an inhibitor of mitochondrial function rather than of DNA replication. The biologic effects were triggered in the first minutes of exposure to the cells when anthralin became rapidly associated with the cell membranes. Labeling experiments with [^{14}C]anthralin revealed that the manifestation of the biologic response occurring after a latency phase of some hours coincided with the accumulation of radioactivity in the *intra*cellular particulate fraction. The cytosol remained essentially unlabeled.

The discovery of the antipsoriatic activity of chrysarobin (1,8-dihydroxy-3-methyl-9-anthrone) [1] and subsequently of anthralin (dithranol, cignolin, 1,8-dihydroxy-9-anthrone) [2,3] has stimulated investigations on the molecular mode of action of this group of antiproliferative compounds. Essentially three different cellular targets are discussed:

1. DNA replication and repair synthesis are inhibited [4,5]. A direct interaction of anthralin with cellular DNA as proposed by Swanbeck and Thyresson [6], however, has been questioned [7,8].
2. Mitochondrial structure and function are affected, leading to inhibition of cellular respiration [9–13].
3. Cytosolic key enzymes are impaired, for example, glucose-6-phosphate dehydrogenase in the hexose monophosphate shunt [14–16]. Since all three effects can independently account for the inhibition of cell growth and proliferation observed after the application of anthralin, we wanted to know which is the most sensitive.

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* Present address: INSERM U 211, Faculté de Médecine, Université de Nantes, Nantes, France.

Reprint requests to: Uwe Reichert, Ph.D., CIRD, Sophia Antipolis, F-06565 Valbonne Cedex France.

Abbreviations:
DMEM: Dulbecco's modified Eagle's medium containing 5.5 mM glucose and 4 mM glutamine
F12: Ham's medium F12
FCS: fetal calf serum
HEPES: 4-(2-hydroxyethyl)-piperazine-1-ethane-2-sulfonic acid
HPLC: high-performance liquid chromatography
IC_{50}: concentration of a given compound causing 50% inhibition of the biologic activity under investigation
METAMED: "metabolism test medium" (DMEM without pyruvate and sodium bicarbonate but with 20 mM HEPES)
TCA: trichloroacetic acid

The studies were performed with the transformed human keratinocyte line SV-K14. Thymidine incorporation was taken as a measure of DNA synthesis [17]. Carbon dioxide release from glutamine was used to determine respiratory activity since we found this amino acid to be one of the most important oxidative energy sources for cultured human keratinocytes [18]. To evaluate the effect of anthralin on the hexose monophosphate pathway we measured CO_2 production from glucose taking advantage from the finding that human epidermal cells in vitro generate about two-thirds of this CO_2 via the shunt and only one-third via the Krebs cycle [19]. On comparing dose response and inhibition kinetics of anthralin on these 3 functions we found respiration to be the most sensitive target. This finding received further support from parallel experiments using inhibitors that are known to act selectively on DNA (mitomycin C) [20] or mitochondria (antimycin A) [21].

MATERIALS AND METHODS

Chemicals

Anthralin and its oxidation products, anthralin-quinone (danthron, 1,8-dihydroxy-anthraquinone) and anthralin-dimer (1,8,1′,8′-tetrahydroxy-10,10′-dianthrone), were kindly provided by Dr. J. Maignan (L'Oréal, Aulnay-sous-Bois) and Dr. B. Shroot (CIRD, Valbonne); [10-^{14}C]anthralin was prepared by Dr. J.-P. Frideling (CIRD, Valbonne). Cationic silica microbeads were a generous gift of Dr. B. Jacobson (University of Massachusetts, Amherst). Antimycin A and mitomycin C were from Boehringer Mannheim. Culture media and fetal calf serum (FCS) were purchased from Flow Laboratories and GIBCO. [Methyl-^{3}H]thymidine, [U-^{14}C]glucose, and [U-^{14}C]glutamine were from CEA, Gif-sur-Yvette.

Stability of the Compounds

The spontaneous oxidation of anthralin in solution was followed by high-performance liquid chromatography (HPLC). At different times aliquots of the solutions were diluted and subjected to HPLC analysis using the chromatographic equipment and the conditions described elsewhere [16].

Cells and Culture Conditions

The SV-40 transformed human foreskin keratinocyte line SV-K14 (a kind gift of Dr. B. Lane, Imperial Cancer Research Fund, London) was used after about 20 passages. The cells were grown at 37°C (5% CO_2, humidified atmosphere) in Dulbecco's modified Eagle's medium containing 5.5 mM glucose and 4 mM glutamine: Ham's medium F12 (DMEM:F12) (1:1) containing 100,000 U penicillin, 100 mg streptomycin, and 250 μg amphotericin B per liter of the medium which was supplemented with 5–10% FCS depending on the batch.

Preparation of the Cells for the Experiments

Forty-eight hours before application of the compounds, 40,000 cells were seeded together with 0.5 ml of the culture medium in each of the 24 wells of a COSTAR Tissue Culture Cluster.

Application of Anthralin and Reference Compounds

Immediately before use, 0.1–10 mM stock solutions of anthralin and its analogs were prepared with glacial acetic acid in which the compounds are stable for several hours. These solutions were diluted a thousandfold with serum-free metabolism test medium (METAMED) and, after neutralization with 10 N NaOH, added in 0.5-ml quantities to each well. The use of serum-free medium was necessary since we

found that anthralin is partially bound and inactivated by serum components.

Mitomycin C was dissolved at 150 μM in METAMED before its application, and 30 mM antimycin A in ethanol was diluted 1500-fold with METAMED.

Assessment of the Biologic Effects

The cells were exposed to the compounds for the time periods indicated. This was done at 37°C in a humidified incubator without CO_2 supply. Thereafter, either 25 μl FCS was added per well or the medium was replaced by fresh METAMED containing 5% serum.

To trace CO_2 evolution from glucose and glutamine, 0.1 μCi [^{14}C] glucose or [^{14}C]glutamine was added to each well. The covers of the clusters were equipped with glass-fiber filters which were soaked with 2 N KOH to trap the [^{14}C]carbon dioxide evolved. At the end of the experiment, the filters were dried and subjected to liquid scintillation counting using Pico-Fluor 30 (Packard Instruments) as the scintillation liquid.

To measure thymidine incorporation into the cells, 0.25 μCi [^{3}H] thymidine was added to each well. The incorporation was stopped by aspirating the medium. The trichloroacetic acid (TCA)-soluble pool was extracted twice with 0.5 ml 5% (w/v) TCA. The remaining material was dissolved in 0.5 N NaOH. Aliquots were taken to measure the tritium content by scintillation counting. The protein content was assayed with the BIORAD method.

Intracellular Distribution of ^{14}C-Label after Application of [^{14}C]Anthralin

Cells were cultured to confluency in 75-cm^2 Falcon tissue culture flasks before the growth medium was replaced by 10 ml serum-free METAMED containing 0.1 μmol [^{14}C]anthralin (39 Ci/mol).

Plasma membranes from the anthralin-treated cells were isolated at 4°C by adapting the density pertubation technique with cationic silica microbeads from *Saccharomyces* [22] and *Dictyostelium* [23] cells to cultured keratinocytes (a full description of this procedure has been submitted for publication). In brief, trypsinized cells were coated with the microbeads (diameter about 50 nm, specific gravity > 2 g/cm^3) and lysed by hypoosmotic shock. The plasma membranes were separated from the cytosol and other cell organelles by low-speed centrifugation (4°C, 5 min, 200 *g*). They were recovered in the pellet (fraction "P1"). The supernatant was then subjected to high-speed centrifugation for 30 min at 50,000 *g* (pellet "P2", supernatant "S"). The whole procedure required about 1 h. The radioactivity in the 3 fractions was determined by liquid scintillation counting, and their protein content was measured with the BIORAD assay.

RESULTS

In a first set of experiments, the cells were exposed for 3 h to different concentrations of anthralin and, in parallel, to its oxidation products, anthralin-dimer and anthralin-quinone (for structures see Fig 2). The treatment was terminated by replacing the drug-containing medium with METAMED supplemented with 5% FCS, [^{3}H]thymidine, and [^{14}C]glutamine or [^{14}C]glucose. After 24 h incubation, protein content, thymidine incorporation, and CO_2 production from glutamine and glucose were measured for each well as described in *Materials and Methods*. They were related to the controls which were set to 100%.

Fig 1 shows the corresponding dose-response curves. Anthralin revealed the strongest inhibition: the dimer was generally less active and the quinone inactive. The dimer inhibited all parameters at IC_{50} values of about 6 μM. The effect of anthralin was more specific: in the average of 11 independent experiments, 50% inhibition was achieved at about 1 μM for the respiration of glutamine, at 3 μM for thymidine incorporation and CO_2 production from glucose, and at 10 μM for the protein content. The IC_{50}'s obtained from different experiments varied between half and double these values. The variations were probably due to the instability of the compounds.

Since the incubation periods chosen for anthralin exposure (3 h) and the expression of the biologic response (24 h) were arbitrary, we compared in a first control experiment the dose-response at 10.5 h and 24 h after a 3-h treatment with anthralin: the respective IC_{50} values were 5.0 μM and 6.5 μM for thymidine

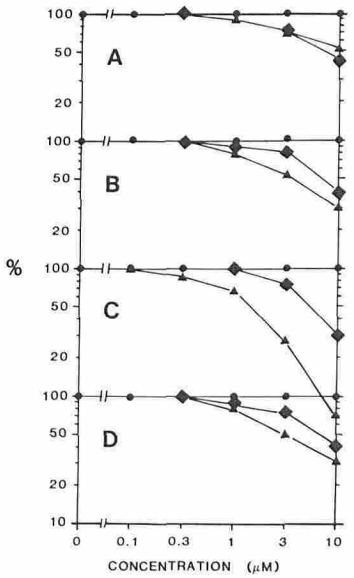

FIG 1. Dose-response curves for the effects of anthralin (▲), anthralin-quinone (●), and anthralin-dimer (◆) on protein content per well (*A*), thymidine incorporation (*B*), glutamine respiration (*C*), and CO_2 production from glucose (*D*). The cells were treated for 3 h with the compounds in the absence of serum. The biologic response was assessed after 24-h incubation in fresh culture medium.

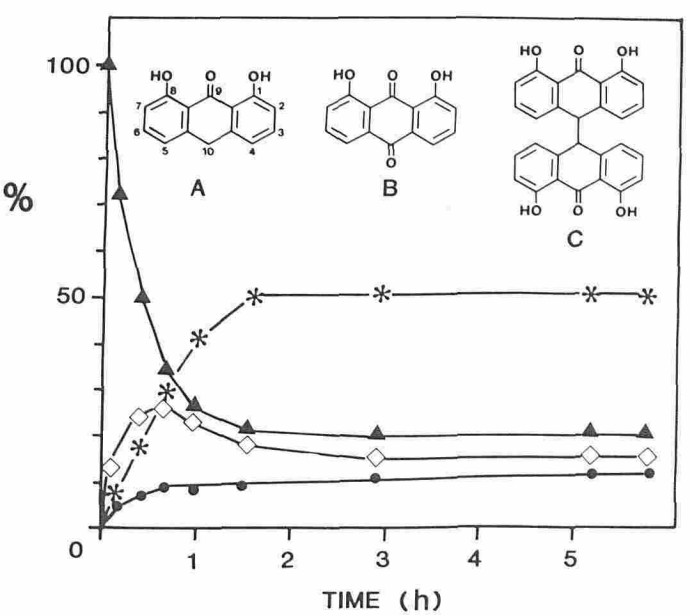

FIG 2. Disappearance of anthralin (initial concentration 1 μM) from serum-free culture medium (▲, structure *A*) by chemical oxidation to anthralin-quinone (●, structure *B*), anthralin-dimer (◇, structure *C*) and to an undefined material ("anthralin-brown", *) which is not detectable by HPLC. The amount of the latter was calculated by subtraction.

incorporation and 0.67 μM and 0.73 μM for glutamine respiration, indicating that this dose-response parameter is relatively insensitive to variations in the response time. In order to obtain more information about (i) the time course of anthralin decomposition in the incubation medium and (ii) the time courses of induction and manifestation of the biologic effects, the following kinetic experiments were performed.

When anthralin was transferred from glacial acetic acid (in which it was stable for several hours) to serum-free culture medium (in which it was applied to the cells), it disappeared exponentially as confirmed by HPLC analysis (Fig 2). After 1–

2 h, a constant level of anthralin (or a comigrating decomposition product) was observed. During this time 2 other derivatives, which were eluted at the positions of anthralin-dimer and anthralin-quinone, appeared. At equilibrium (about 3 h after dilution), the detectable molecular species amounted to 48% of the initial anthralin concentration. They were recovered as 20% "anthralin," 16% "dimer," and 12% "quinone." The other 52% was converted to an undetectable oxidation product, most probably "anthralin brown," which was retained by the column.

In the next experiment, the cells were exposed to a fixed concentration of anthralin (3 μM) for different times. The expression of the biologic response was determined 24 h later. As shown in Fig 3, an incubation time of 15 min was sufficient to trigger most of the biologic effects under investigation. Only the maximum inhibition of CO_2 production from glucose required a longer incubation. In this particular experiment, thymidine incorporation was the least affected parameter, followed by the protein content per well, and the conversion of glucose to CO_2. In agreement with the first experiment, respiration of glutamine reacted with the strongest inhibition.

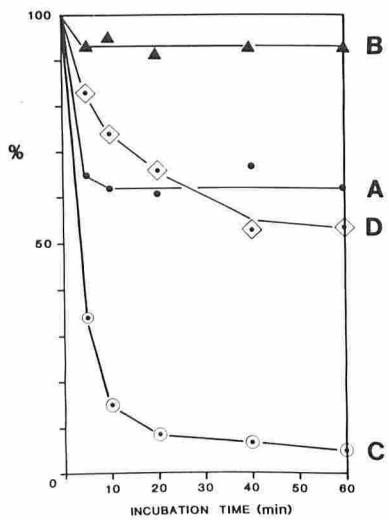

FIG 3. The time dependency of anthralin action on protein content per well (*A*), thymidine incorporation (*B*), glutamine respiration (*C*), and CO_2 production from glucose (*D*). The cells were treated for different times with 3 μM anthralin in serum-free medium. The biologic response was assessed after 24-h incubation in fresh culture medium.

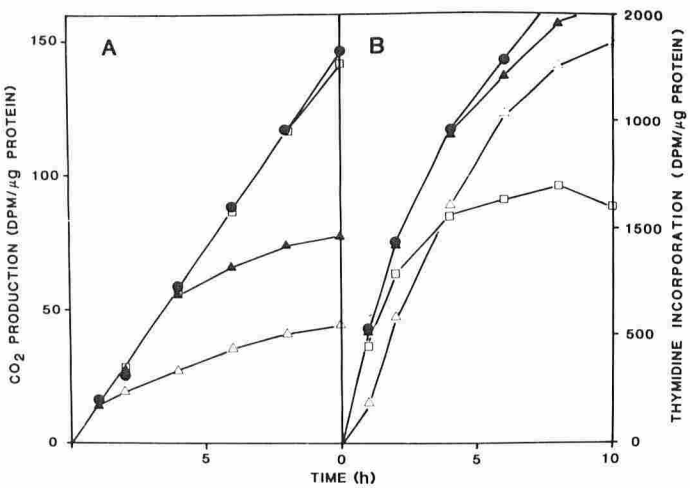

FIG 4. Carbon dioxide production from glutamine (*A*) and thymidine (*B*) incorporation in the absence (●) and in the presence of 3 μM anthralin (▲), 20 μM antimycin A (△), and 150 μM mitomycin C (□). The compounds were added at zero time in serum-free medium; the medium was supplemented after 1 h with 5% (v/v) FCS.

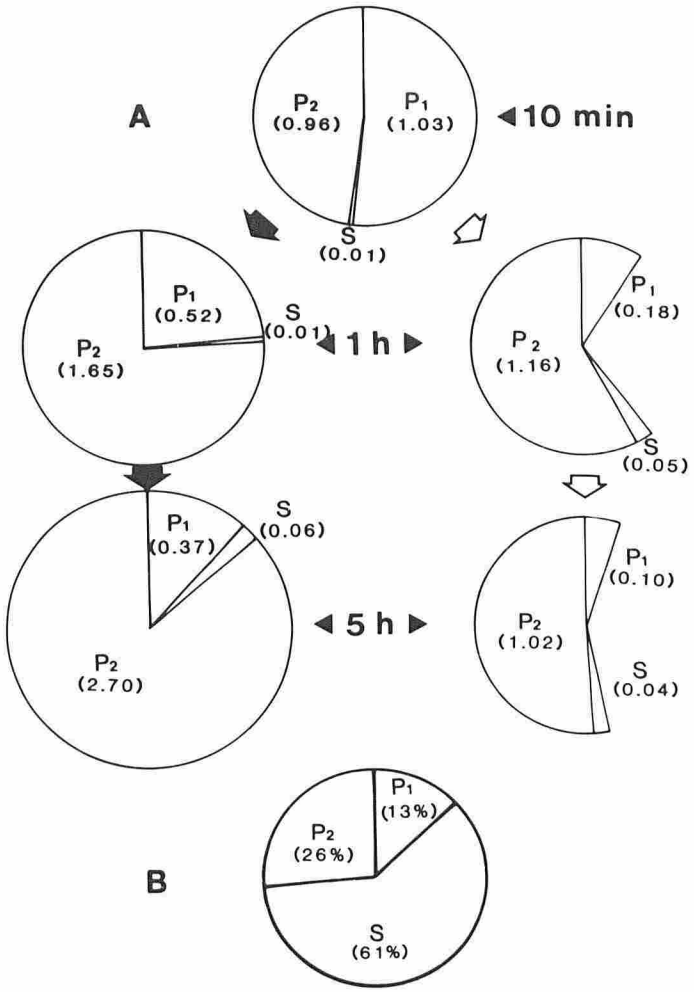

FIG 5. Intracellular distribution of the radioactivity from [^{14}C]anthralin (*A*) and the distribution of proteins (*B*) in 3 cellular subfractions. *P1* = plasma membrane fraction; *P2* = intracellular particulate fraction (mainly mitochondria and lysosomes); *S* = 50,000 *g* supernatant (mainly cytosol). The cells were incubated either for 10 min, 1 h, or 5 h with 10 μM [^{14}C]anthralin in serum-free medium (left side) or for 10 min with [^{14}C]anthralin followed by an incubation in fresh serum-containing medium (right side). The areas of the circles are proportional to the total radioactivity incorporated into the cells. The *open sectors* correspond to the radioactivity removable by serum-containing medium. *In parentheses*, [^{14}C]concentrations are given (fmol/cell).

The following experiment was designed to estimate the time necessary for the manifestation of the biologic response after the application of 3 μM anthralin. We restricted ourselves to the study of thymidine incorporation and glutamine respiration, but included 2 other compounds that are known to act specifically on either DNA or respiration: mitomycin C, which is presumed to interact with guanine residues thereby inhibiting DNA replication [20], and antimycin A, which interrupts the electron transport chain between cytochromes b and c_1 [21]. In contrast to the other experiments, the cells were preincubated for 3 h with the radioactive glutamine before adding inhibitors and [^{3}H]thymidine since we found that the evolution of [^{14}C] carbon dioxide from [^{14}C]glutamine occurred with a lag phase of about this duration. After 1-h exposure, 25 μl FCS was added to each well. [^{14}C]carbon dioxide evolution and [^{3}H]thymidine incorporation were monitored over the next 9 h.

Fig 4*A* shows that respiration was affected 1 h after the addition of 20 μM antimycin and 4 h after the addition of 3 μM anthralin. This inhibition was accompanied by an evident but less pronounced reduction in thymidine incorporation (Fig 4*B*).

Mitomycin at 150 μM, on the other hand, needed 1 h to reduce the incorporation of thymidine but 8 h to affect respiration.

Since the experiments described above indicated that respiration is the primary target of anthralin action and since the last 2 experiments showed clearly that there is a latency phase between the triggering of the biologic response and its manifestation, it was tempting to correlate this phase with the lag time of the drug (or its reactive conversion product) to appear in the mitochondria. For this reason we measured the kinetics of the intracellular distribution of ^{14}C-label from [10-^{14}C]anthralin.

The cells were incubated for different times with 10 μM [^{14}C] anthralin at 37°C in the absence of serum. Thereafter, 3 cellular subfractions were prepared as described in *Materials and Methods*. They consisted predominantly of plasma membranes (fraction P1), mitochondria and lysosomes (fraction P2) [24], cytosol and light particulate material (fraction S). Nuclei were largely destroyed during membrane preparation and were presumed to contribute mainly to fraction S.

The results of the experiment are given in Fig 5*A* (left side). After 10 min, 1 h, and 5 h of incubation, 2.00, 2.18, and 3.13 fmol [^{14}C]carbon per cell, respectively, were recovered, corresponding to 7.5, 8.2, and 11.8% of the radioactivity supplied. After the 10-min incubation, more than 50% of the label was found in the plasma membranes, the rest in fraction P2. With increasing time, the radioactivity was successively translocated from fraction P1 to fraction P2 which, after 5 h, contained almost 90% of the label. The supernatant remained essentially free of radioactivity.

In a parallel experiment, cells were treated as before for 10 min with [^{14}C]anthralin. After aspirating the drug-containing medium, the cells were kept 1–5 h in METAMED supplemented with 5% FCS before fractionation. During this time, 30–40% of the radioactivity incorporated within the first 10 min was released—mainly from the plasma membranes—into the medium. The total amount of bound radioactivity decreased from 2.00 via 1.39 to 1.16 fmol/cell (Fig 5*A*, right side).

Both experiments led to a predominant accumulation of the cell-bound radioactivity in fraction P2. This was not correlated with the distribution of proteins (Fig 5*B*), indicating that the preferred association of anthralin (or its conversion products) with this fraction cannot be explained by an unspecific binding to proteins.

DISCUSSION

Anthralin inhibits different cellular functions at rather low concentrations. The following IC_{50} values, for example, are reported in the literature: 1.1 μM and 2.3 μM for the inhibition of DNA replication and repair synthesis in T98G cells [5], 0.3 μM and 1.5 μM for the inhibition of thymidine incorporation and respiration in cultured human dermal fibroblasts [25,26], and about 50 μM for the inhibition of purified yeast glucose-6-phosphate dehydrogenase [15] or mouse epidermal arachidonic acid lipoxygenase [27] activity.

From the above data obtained with fibroblasts, thymidine incorporation appears to be more sensitive to anthralin than to respiration. This interpretation, however, can be misleading when the experiments are not conducted under identical conditions. In the experiments cited, for example, thymidine incorporation was studied in actively growing cells, whereas respiration was measured with confluent cultures in order to produce a stronger signal. To avoid this ambiguity, we applied, in the present paper, exactly the same conditions for the measurement of all parameters. Furthermore, we used human epidermal keratinocytes instead of other cell types because they represent a likely target cell of anthralin action in psoriasis.

All our results obtained under such conditions indicate that the inhibition of cell proliferation by anthralin, at least in keratinocytes, is the consequence of impaired mitochondrial function rather than of DNA damage.

Furthermore, the present study shows that when keratinocytes are exposed to [^{14}C]anthralin, a certain percentage of the label is immediately adsorbed on their plasma membranes, later becoming redistributed over the intracellular particulate fraction (Fig 5). This rapid process, which occurs during the first 10 min and can be partially reversed by the addition of serum-containing medium, is followed by a further, much slower uptake of anthralin or one of its oxidation products. The first, rapid process is mainly responsible for the initiation of the biologic response since incubation times longer than 15 min do not give rise to quantitative changes of most of the parameters measured. This finding is in agreement with the report of Zetterberg [28] that, in yeast, the maximal induction of respiration-deficient "petites" colonies by anthralin was reached within 5 min.

Whereas the inhibition kinetics of thymidine incorporation, protein content, and glutamine respiration can be considered as monophasic, the inhibition of CO_2 production from glucose appears to be biphasic (Fig 3). This peculiarity can easily be explained by the fact that part of the CO_2 production occurs via the Krebs cycle and the rest via the hexose monophosphate shunt [19], and by the assumption that the inhibition of these pathways follows a different time course. Further experiments, however, are necessary to clarify this point.

In any case, we can state that the rapid association of anthralin with the plasma membrane triggers most of the biologic effects which become manifest some hours later (Fig 4). The time lag between induction and expression of these effects can be explained, at least in part, by the intracellular redistribution of the reactive species, i.e., anthralin itself or one of its cellular conversion products.

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